REMARKS

Claims 5-11 and 14-16 are pending in the present application.

Applicants wish to thank Examiner Tran for the helpful and courteous discussion with their undersigned Representative on December 2, 2003. During this discussion, several amendments and arguments were discussed to overcome the objections to the claims, the written description rejection, and the rejection over the art of record. The content of this discussion is reflected by the amendments and remarks set forth herein.

The rejection of Claims 3, 5-13, and 15-16 under 35 U.S.C. §112, first paragraph ("written description") is traversed.

The Office has alleged that the specification fails to meet the written description requirement of 35 U.S.C. §112, first paragraph (paper number 18, page 4). Applicants respectfully disagree.

The Office asserts that the specification fails to provide a sufficient description of the scope of the drug. Applicants wish to note, however, that the present invention provides a screening method for *identifying* drug-protein binding partners and the genes encoding the protein. As fully described on pages 3-5, Applicants have created a tri-functional probe having a drug, a chemical cross-linker, and an antigenic substance (reporter) for use in the inventive method. Accordingly, Applicants wish to draw the Examiner's attention to the fact that the identity of the drug is not intended to be limited as the claimed method is *amenable to any drug* so long as it is non-protein and *per se* exhibits no antigenicity, as well as having a group that is reactive with the chemical cross-linker selected. Moreover, the skilled artisan would immediately envisage the scope of alternatives when the chemical cross-linker is selected from the group set forth in previously pending Claim 13 (presently Claim 5).

Applicants remind the Examiner that MPEP §2163.02 defines the written description requirement, stating:

> An objective standard for determining compliance with the written description requirement is, "does the description clearly allow persons of ordinary skill in the art to recognize that he or she invented what is claimed." *In re Gostelli*, 872 F.2d 1008, 1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989).

Applicants submit that this objective standard has been met by the present specification. Specifically, Applicants claim a general method for in vitro detection of a gene encoding a drug-targeted protein. As stated on page 3, lines 10-16, the drug is defined as being suitable for administration to a living organism, which non-protein and has no per se antigenicity. Further, on page 4, lines 1-7, Applicants discuss the relevance and necessity of having or introducing a functional group that is compatible with the selected cross-linker, as well as the need to conserve the efficacy of the drug when coupled with the cross-linker. The structure of the drug itself is not particularly relevant, so long as it meets these criteria outlined above. Moreover, the proteins that are targeted by the drug that is selected is directly correlated to the drug selected and since the claimed method seeks to identify these very proteins, the Examiner's objection based on the lack of recited structure, function, or cellular location is of no moment.

Therefore, the present claims do clearly allow the skilled artisan to recognize what has been invented and what is claimed is adequately described in the specification within the meaning of 35 U.S.C. §112, first paragraph. Accordingly, withdrawal of this ground of rejection is requested.

The rejection of Claims 3, 5, 7, and 15 under 35 U.S.C. §102(b) over Gram et al is obviated by amendment.

As asserted by the Examiner, Gram et al disclose a method for in vitro detection of a gene encoding a drug-targeted protein. However, as conceded by the Examiner, Gram et al is silent with respect to the specific cross-linking agents in previously pending Claim 13. Claim 5 has been amended to recite these cross-linking agents and, as such, is now free from the disclosure of Gram et al.

Accordingly, Applicants request withdrawal of this ground of rejection.

The objections to Claim 3 are obviated by cancellation of this claim. Turning to the objection based on the numbering of the claims, Applicants wish to draw the Examiner's attention to the fact that the objected to numbering arose due to previous amendments. Applicants remind the Examiner that it is her duty to renumber the claims once the application has been allowed (37 C.F.R. §1.126). Accordingly, no further amendment by the Applicant is believed to be necessary.

Acknowledgment that these objections have been withdrawn is requested.

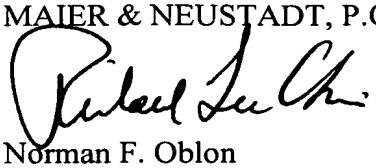
Finally, during the discussion there was question as to the proper recitation of the cross-linker "sulfosuccinimidyl-4-(p-maleimidophenyl) butyrate" in Claims 13 and 14. Applicants **submit herewith** Iwai et al and page 330 of the Pierce Catalog evidencing that "sulfosuccinimidyl-4-(p-maleimidophenyl) butyrate" is proper. Accordingly, Claim 14 has been amended to recite this proper chemical name.

Applicants submit that the present application is now in condition for allowance.

Early notification of such action is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.



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ANALYTICAL BIOCHEMISTRY 171, 277–282 (1988)

Preparation of a Verifiable Peptide–Protein Immunogen: Direction-Controlled Conjugation of a Synthetic Fragment of the Monitor Peptide with Myoglobin and Application for Sequence Analysis

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A useful method for preparing a synthetic peptide-carrying protein for specific antibody production was established. The monitor peptide is a trypsin-sensitive cholecystokinin-releasing peptide purified from rat pancreatic juice on the basis of its stimulatory activity toward pancreatic enzyme secretion. The NH_2-terminus fragment of the monitor peptide (residues 1–14) was synthesized by a solid phase method. Cysteine at the COOH terminus of the fragment was conjugated with amino groups of myoglobin using a hetero-bifunctional reagent. Sequence analysis of the fragment–myoglobin conjugate indicated that the peptide/myoglobin conjugation ratio was about 1/1 (mol/mol). Antiserum against the conjugate from a rabbit effectively abolished the stimulatory activity of the monitor peptide in the rat small intestine.

The monitor peptide is a trypsin-sensitive cholecystokinin (CCK)[2]-releasing peptide that was purified from rat pancreatic juice on the basis of its stimulatory activity toward pancreatic enzyme secretion and named according to its physiological function in the small intestine (1–4). The monitor peptide originates from pancreatic acinar cells (5) and shows multiple biological activities including CCK-releasing activity (6), cell-growth-stimulating activity (7), and trypsin inhibitory activity (4).

For further biochemical and physiological investigations of the monitor peptide, it is necessary to establish a specific and sensitive method for immunological quantitation of the monitor peptide, because the detection of the monitor peptide depends on a bioassay with the intact rat, and the assay of trypsin inhibitory activity is not so specific.

Sequence analysis indicated that the monitor peptide comprises 61 amino acids, and its middle region (residues 21–40) shows high homology with that of a Kazal-type trypsin inhibitor (pancreatic secretory trypsin inhibitor, PSTI) (4). These results suggest that the monitor peptide should be classified as a Kazal-type inhibitor and that it may be a rat pancreatic secretory trypsin inhibitor or a related peptide. Its unique 61-amino acid sequence is, however, less related to those of other mammalian PSTIs than the latter are to each other (4).

In order to obtain region-specific antiserum against the monitor peptide, we establish here a useful method for preparing a synthetic peptide-fragment-carrying myoglobin as an antigen. We chose the N-terminal re-

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[2] Abbreviations used: CCK, cholecystokinin; PSTI, pancreatic secretory trypsin inhibitor; SMPB, succinimidyl 4-(*p*-maleimidophenyl) butyrate; ACN, acetonitrile; TFA, trifluoroacetate; PTH, phenylthiohydantoin.

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gion of the monitor peptide, residues 1–14, which shows less homology with those of other related peptides.

MATERIALS AND METHODS

Materials. Sperm whale myoglobin was obtained from Sigma. Succinimidyl 4-(*p*-maleimidophenyl) butyrate (SMPB) was obtained from Pierce. 1;4-Dioxane was obtained from Wako Chemicals. Acetonitrile (ACN) and trifluoroacetate (TFA) were purchased from Applied Biosystems, Inc. (ABI).

Synthesis of a peptide fragment. A fragment (residues 1–14) of the monitor peptide, Gly–Asn–Pro–Pro–Ala–Glu–Val–Asn–Gly–Lys–Thr–Pro–Asn–Cys, was synthesized with an ABI Model 430A peptide synthesizer. The primary amino acid sequence of monitor peptide and the region of the synthesized fragment, the latter being underlined, are shown in Fig. 1. Purification of the synthesized fragment was performed by HPLC (ABI 130A separation system, with a 0.21 × 3-cm column containing C_8, 300 A pore solid support). The purity was determined with an ABI 477A protein sequencer (8).

Introduction of SMPB into myoglobin. Two milligrams of myoglobin was dissolved in 2 ml of 0.1 M sodium phosphate, pH 8.0. The insoluble material was removed from the solution by centrifugation (2000*g*, 10 min). Three milligrams of SMPB was dissolved in 1 ml of 1,4-Dioxane. Two milliliters of the myoglobin solution and 1 ml of the SMPB solution were mixed. The reaction was carried out for 10 min with gentle stirring. Then the reaction mixture was immediately loaded onto a Sephadex LH 20 column (1.8 × 26 cm) which had been equilibrated with 50% (v/v) ACN containing 0.0005% TFA (pH 3.9), and the SMPB–myoglobin fractions obtained were pooled.

Conjugation of SMPB–myoglobin with the synthetic peptide. One milligram of the peptide fragment was dissolved in 1 ml of the solvent used for the gel chromatography. The peptide fragment solution was added to the SMPB–myoglobin fraction eluted from the column. The reaction was carried out for 1 h with gentle stirring. The fragment–myoglobin conjugate was purified by HPLC.

Determination of the contents of the conjugate preparation. The purified peptide fragment–myoglobin conjugate was sequenced with an ABI 477A protein sequencer.

Preparation of antiserum against the peptide fragment–myoglobin conjugate. Immunization was carried out according to the method described in Ref. (9). One milligram of the conjugate in 0.5 ml of distilled water was emulsified with 0.5 ml of Freund's complete adjuvant. Small portions of the resultant emulsion were injected into different sites on the back the footpad of the rabbit subcutaneously. The rabbit was given a booster of 1 mg of the conjugate without the adjuvant after 4 weeks and then bled 10 days after the booster shot. The animal was used every 1 or 2 months to obtain the blood 10 days after each booster shot. Plasma was obtained and stored at −60°C until use.

Neutralization of the stimulatory activity of the monitor peptide by the antiserum. Nine micrograms of the purified monitor peptide was incubated at 37°C with 5 ml of the antiserum, from a rabbit immunized with the synthetic fragment–myoglobin conjugate, for 3 h. Then 1.25 ml of the mixture (2.25 μg of the monitor peptide) was infused into an anesthetized rat duodenum. The experimental method used was described in Ref. (3). Briefly, a rat (Wistar male, 300 g) was anesthetized with pentobarbital for insertion of bile–pancreatic and duodenal cannulae. The rat's small intestine was infused with saline containing 0.1 mg/ml of soybean trypsin inhibitor to wash out intraluminal protease, which resulted in a protease-free intestine. Then the sample to be tested was injected into duodenum via the duodenum cannula and secreted bile–pancreatic juice was collected via the bile–pancreatic cannula. Pancreatic enzyme secretion in response to infu-

sion of the sample was determined by measurement of the trypsin activity in the bile-pancreatic juice collected.

As a control, 9 μg of the monitor peptide was incubated under the same conditions as above except with 5 ml of control antiserum from a rabbit immunized with lactoalbumin. This was a control to determine whether nonspecific antibodies and other substances in serum could interfere with the pancreatic enzyme secretion stimulated by the monitor peptide. Then, 1.25 ml of the mixture was infused into the rat duodenum and pancreatic enzyme secretion was determined from the trypsin(ogen) output.

Determination of trypsin activity. Trypsin activity was determined using benzoyl-arginine-*p*-nitroanilide as a substrate (10) after activation with porcine enterokinase (Sigma) at 37°C for 40 min (11).

RESULTS AND DISCUSSION

Using a hetero-bifunctional reagent, SMPB, we successfully conjugated the syn-

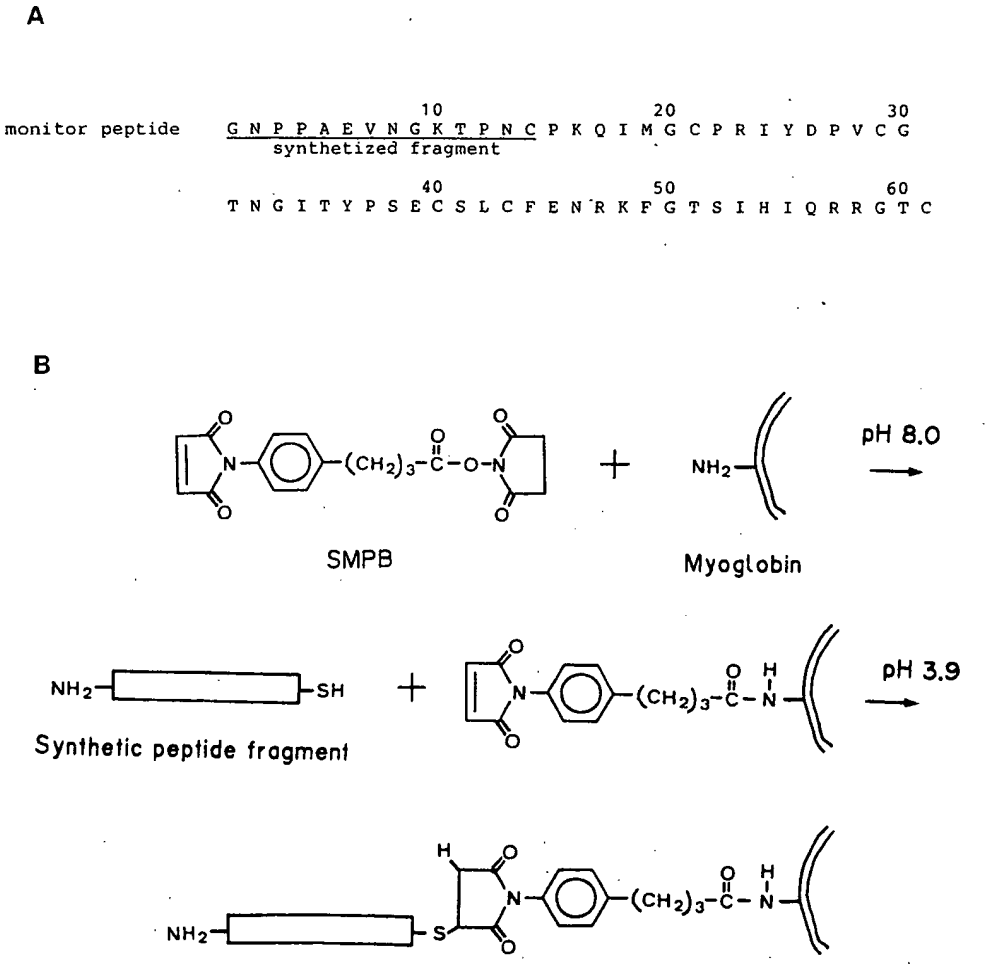

FIG. 1. (A) Amino acid sequence of the monitor peptide (from Ref. (4)). The region corresponding to the synthesized fragment is underlined. The one letter amino acid abbreviations are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr. (B) Schematic diagram of the conjugation of the synthetic fragment with myoglobin using SMPB.

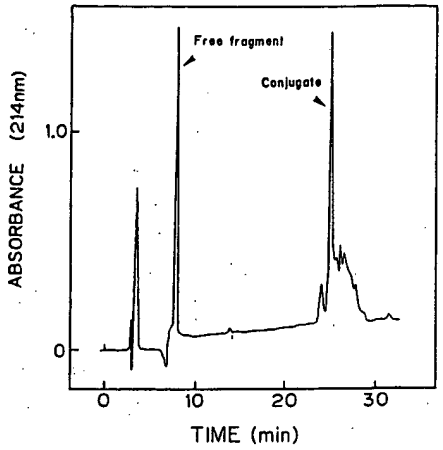

FIG. 2. Purification of the synthetic fragment–myoglobin conjugate by HPLC (ABI 130A separation system). Separation conditions are as follows. Sample: A portion of the reaction mixture of the synthetic fragment and SMPB–myoglobin. Column: Aquapore RP300 (C_8, 300 A), 2.1 × 30 mm. Flow rate: 0.2 ml/min. Solvent system: (A) 0.1% trifluoroacetic acid, (B) 0.1% trifluoroacetic acid:acetonitorile, 30:70 (v/v). Initial condition of 100% (A) to 50% (A) and 50% (B) for 20 min with a linear gradient then an isocratic elution with 100% (B).

thetic peptide fragment with myoglobin, with control of the reaction, with a high yield.

Although the succinimidoester of SMPB readily reacts with the primary amino groups of a carrier protein at pH 8.0, the maleimido groups of the SMPB–protein conjugate are very unstable at this pH. Twenty minutes after the SMPB had been reacted with myoglobin at pH 8.0, the SMPB–myoglobin could no longer react with the SH groups of the peptide fragment, and when the same reaction was performed in a buffer of pH 6.2, the efficiency of the conjugate formation was very low (data not shown).

Therefore, in our experiments, SMPB was reacted with myoglobin at pH 8.0, the pH was immediately lowered to 3.9 for gel filtration, and free SMPB was separated from the SMPB–myoglobin at the same time. Then the peptide fragment was conjugated with SMPB–myoglobin at pH 3.9 for an efficient reaction. The peptide–myoglobin conjugate gave a single peak on HPLC, as shown in Fig. 2.

We chose whale myoglobin as a carrier protein because it is a small red-colored protein (M_r 17,000) which is readily soluble in both water and 1,4-Dioxane, and its amino acid sequence has been completely determined (12). These points make interpretation of the results of sequence analysis of the products reliable. Furthermore, myoglobin contains no cysteine, which is beneficial for both directing the succinimidoester of SMPB to the target and avoiding self-aggregation of the SMPB–protein. The synthetic fragment–myoglobin conjugate was analyzed with a gas phase protein sequencer. In the first cycle of the sequencing, 68.6 pmol of PTH–glycine and 19.2 pmol of PTH–valine were detected, as shown in Fig. 3. In the second cycle, PTH–asparagine and PTH–leucine were detected. The sequencing was continued up to the 15th cycle, and the major sequence was found to be Gly–Asn–Pro–Pro–Ala–Glu–Val–Asn–Gly–Lys–Thr–Pro–Asn, which corresponded to that of the synthesized fragment. The minor sequence was determined to be Val–Leu–Ser–Glu–Gly–Glu–Trp–

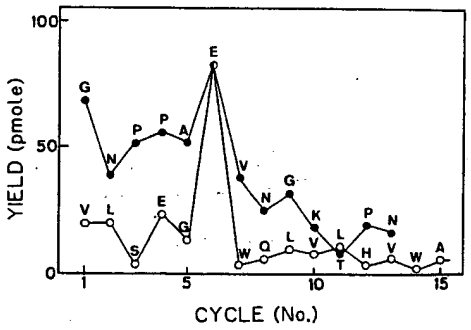

FIG. 3. Amino acid sequencing of the synthesized fragment–myoglobin conjugate. About 100 pmol of the conjugate was loaded onto a protein sequencer 477A (ABI), and its sequence was determined up to residue 15. The yields of PTH–amino acids are shown for each Edman degradation cycle. In the sixth cycle, only Glu was detected. The amino acid sequences corresponding to those of the fragment (○) and myoglobin (●) are indicated by lines in the figure.

Gln–Leu–Val–Leu–His–Val–Trp–Ala–. In the 6th cycle, glutamic acid was found to be a common residue in the fragment and the myoglobin. The minor sequence was identified as the N-terminus of myoglobin. In the 14th cycle, a trace of tryptophan was detected, and on further analysis only one sequence which could be identified as part of that of myoglobin was detected. The slopes of the repetitive yields of the sequences were roughly parallel.

The low yield of the N-terminus of myoglobin may be due to both the lower reactivity of the N-terminal of intact myoglobin and the blokage of the N-terminal amino group by SMPB. To investigate this phenomenon and to obtain an appropriate coefficient for calculating the reaction efficiency, we performed the same experiment with 2-mercaptoethanol instead of the peptide fragment. Myoglobin (0.5 mg) was modified with SMPB and then the residual maleimido groups were blocked with excess mercaptoethanol after the SMPB–myoglobin had been purified by HPLC. The conjugate (276 pmol) was loaded onto the sequencer, 34 pmol of PTH–valine being recovered as the N-terminal amino acid. This value, 34 pmol, corresponds to 51 ~ 63 pmol after correction supposed for the HPLC and sequencer yields (54 ~ 67%). Therefore, 77 ~ 82% of the N-terminal amino groups of myoglobin can react with SMPB:

$$[1 - (51 \sim 63)/276] \times 100$$

$$= 77 \sim 82(\%), \quad \text{average } 80(\%).$$

The amount of PTH–valine detected in the first cycle was 19.2 pmol in the case of the fragment–myoglobin conjugate, so there was calculated to be 96 pmol of myoglobin:

$$19.2/(1 - 0.8) = 96(\text{pmol}).$$

While the amount of glycine detected for the synthetic fragment–myoglobin conjugate was 68.6 pmol in the first cycle, it was 102 ~ 127 (average 115) pmol after correction for the HPLC and sequencer yields. These results indicate that the conjugate consists of one (or two) peptide fragment and one myoglobin molecule:

$$115/96 = 1.20.$$

The peptide fragment–myoglobin conjugate could be used to effectively induce a functional rabbit antiserum. Preincubation of the monitor peptide with the antiserum diminished the stimulatory activity of monitor peptide toward rat pancreatic enzyme secretion, as shown in Fig. 4.

In summary, we established here a useful method for preparing a synthetic peptide fragment–protein conjugate for antiserum production. In particular, in this study, we optimized the conjugation between the peptide and the SMPB–protein. This will facilitate the production of region-specific antisera against small fragments of biologically active proteins.

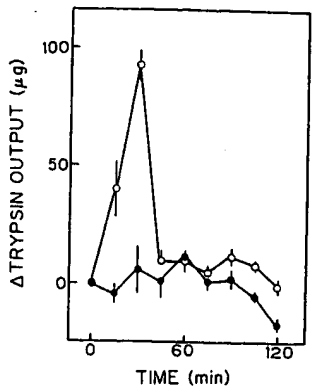

FIG. 4. Pancreatic enzyme secretion in rats in response to intraluminal monitor peptide with or without pretreatment with the specific antiserum obtained using the fragment–myoglobin conjugate as an antigen. In a control study (○), 9 μg of the monitor peptide preincubated with a control serum was infused into the anesthetized rat intestine at 0 time. The same experiment was performed using the antiserum against the synthetized fragment–myoglobin conjugate (●). The net increase in trypsin output (increase from the value at 0 time) is indicated at each sampling time. The values are means ± SEM for four to six rats.

ACKNOWLEDGMENTS

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Note added in proof. Confirmation of the complete amino acid sequence of the monitor peptide has been made, residue 60, Gly, having been revised to Thr. (Iwai, K. *et al.* submitted).

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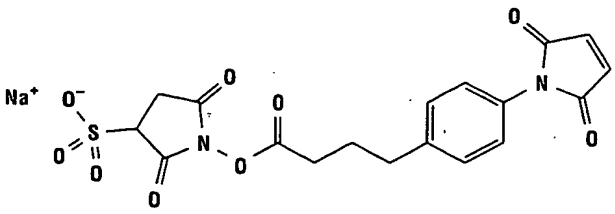
Sulfo-SMPB

Extended chain length analog of Sulfo-MBS.



Sulfo-SMPB
M.W. 458.38
Spacer Arm 14.5 Å

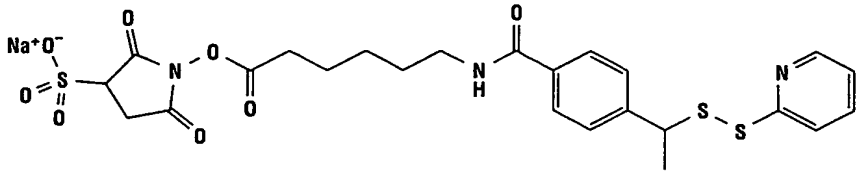
Features/Benefits:
- Extended chain length limits steric hindrance
- Water-soluble; non-cleavable
- Membrane-impermeable
- *Reactive groups:* Sulfo-NHS ester and maleimide
- *Reactive toward:* amino and sulfhydryl groups
- Literature reference #'s 36, 47 (page 342)

Ordering Information

Product #	Description	Pkg. Size
22317	Sulfo-SMPB (Sulfosuccinimidyl 4-[*p*-maleimidophenyl]-butyrate)	50 mg

Sulfo-LC-SMPT

***Form cleavable immunotoxins with greater stability* in vivo.**



Sulfo-LC-SMPT
M.W. 603.67
Spacer Arm 20.0 Å

Features/Benefits:
- Contains a hindered disulfide bond; has formed immunotoxins with improved stability
- *In vitro,* SMPT conjugates are as effective as conjugates formed with SPDP and 2-Iminothiolane
- Does not require exposing the antibody to reducing agents
- Offers an extended spacer arm and water solubility
- *Reactive groups:* Sulfo-NHS ester and pyridyldithio
- *Reactive toward:* amino and sulfhydryl groups
- Literature reference #48 (page 342)

Ordering Information

Product #	Description	Pkg. Size
21568	Sulfo-LC-SMPT (Sulfosuccinimidyl-6-[α-methyl-α-(2-pyridyldithio)toluamido]hexanoate)	50 mg